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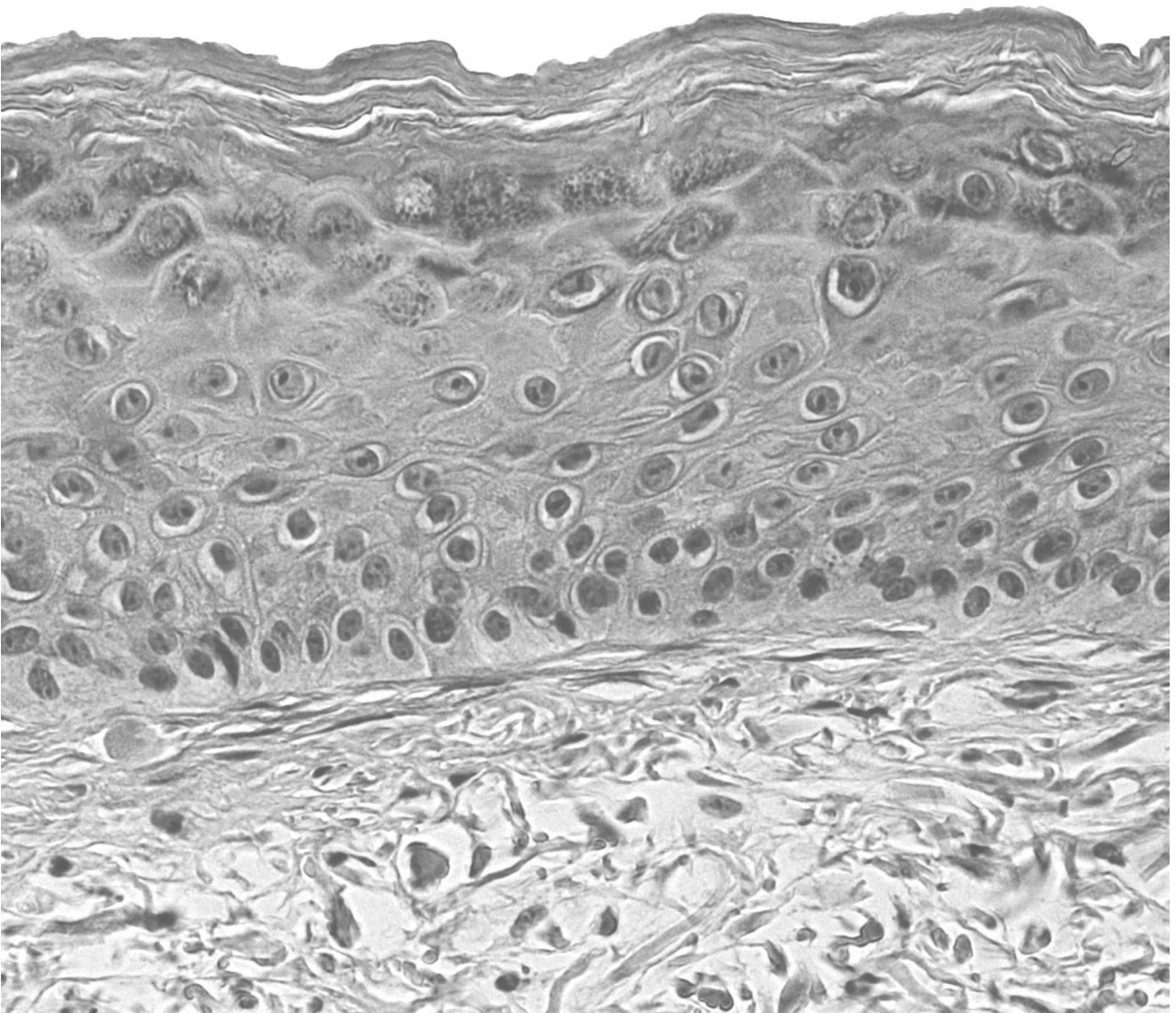
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Chapter 9

Summary and general discussion



SUMMARY AND GENERAL DISCUSSION

Cutaneous wound healing is a dynamic multicellular process which occurs in response to tissue injury. The healed skin results in a scar and the quality of the final scar is a major outcome parameter in healing. The most desirable scar, a normotrophic scar, is thin, flat, barely visible and is mostly seen after superficial injury. However, in predisposed individuals or after extensive trauma, scar formation can result in increased fibrosis, which in turn can result in a hypertrophic scar or a keloid. Both types of scars can severely affect the quality of life of the patients due to physiological and psychological problems^{1,2}. In order to develop treatment strategies for abnormal scars it is important to understand the pathology underlying these abnormal scars. Despite all the research in the field of scar formation, the pathogenesis of hypertrophic scar formation and keloid remains largely unknown^{3,4}. This is partly due to the lack of physiologically relevant human *in vivo*-like abnormal scar models⁵⁻⁸. Such models are important to study the pathogenesis of abnormal scar formation, identify new drug targets and to test new therapeutics. In this study suitable cell types and biomarkers involved in hypertrophic scar formation were identified for use in an *in vitro* hypertrophic scar model. The aim was to develop and validate an *in vitro* full-thickness human tissue-engineered hypertrophic scar model.

In chapter 2 we studied the expression of genes and proteins involved in inflammation, angiogenesis and extracellular matrix (ECM) formation in patients forming hypertrophic scars rather than normotrophic scars. We found that hypertrophic scar formation coincided with a prolonged decrease in expression of inflammatory (TNF α , IL-1 α , IL-1RN, CCL2, CCL3, CXCL2, CXCR2, C3 and IL-10) and angiogenic (ANGPT1, FGF2 and YWHAZ) genes. This coincided with decreased expression of inflammatory mediators and a delayed infiltration of macrophage type 2 cells in hypertrophic scar tissue compared to normotrophic scar tissue. In contrast, there was an extended increased expression of ECM related (Col3A1, TGF β 3 and PLAU) genes during hypertrophic compared to normotrophic scar formation. The decreased expression of pro-inflammatory and inflammatory genes during hypertrophic compared to normotrophic scar formation suggests an immune suppressive rather than immune stimulatory innate immune response and therefore would explain in part the failure of immunosuppressive therapies to improve hypertrophic scar formation. Instead of the currently used anti-inflammatory scar therapies, immune stimulatory therapies may be a better strategy for improving scar quality. Our data also points out that human scar formation following wound healing is a dynamic process that needs to be studied over a period of time rather than a single point in time.

Early in the project it was realised that adipose tissue-derived mesencymal stem cells (ASC) may contribute more to hypertrophic scar formation than their dermal counterparts⁹⁻¹¹. The relevance of using ASC in tissue-engineered scar models is further supported by the fact that ASC are present in the deep cutaneous wound bed which is exposed after 3rd degree burning and which most frequently results in a hypertrophic scar forming¹². Therefore in chapter 3 we studied the response of ASC, dermal fibroblasts and keratinocytes when they come in contact with burn wound exudates which mimics the deep cutaneous burn wound bed in order to determine their importance for tissue-engineered scar constructs and wound healing. Burn wound exudates isolated from human 3rd degree burn wounds were found to contain several mediators (e.g. CXCL8, bFGF, CCL27) related to inflammation and wound healing, which possibly can activate incoming (wound healing) or transplanted cells. Monolayers of ACS and dermal fibroblasts exposed to burn wound exudates both increased secretion of mediators involved in inflammation and wound healing (CXCL1, CXCL8, CCL2, CCL20), but only ASC responded by increasing secretion of angiogenic factor VEGF and wound healing mediator IL-6. This may indicate that both cells will stimulate inflammation and granulation tissue formation when applied to burn wound bed, but that ASC are more potent in stimulating angiogenesis and wound healing than dermal fibroblasts. Although it should be noticed that when ASC and dermal fibroblasts were incorporated into bi-layered skin substitutes the differences were less pronounced than in monolayers. This may be due to the high mediator release of skin substitutes caused by synergistic crosstalk between keratinocytes and MSC¹³, indicating that skin substitutes may be more beneficial for wounds where activation of the inert wound bed is essential for healing. This is in line with results that skin substitutes containing dermal fibroblasts are suitable for healing chronic wounds¹⁴. Our data indicates that ASC may be even more suited for the use in skin substitutes designed for chronic wounds than dermal fibroblasts. The differential response of ASC and dermal fibroblasts was also seen when monolayers of cells were exposed to rh-CCL27 a skin specific chemokine present in burn wound exudates (chapter 3). From an evolutionary point of view, the difference between ASC and dermal fibroblasts may be considered to be related to the function of the ASC, which is to close life-threatening deep wounds rapidly by strongly promoting the formation of granulation tissue and angiogenesis even at the expense of abnormal scar formation. Keratinocytes did not respond by increasing secretion of mediators involved in wound healing but they showed increased proliferation and migration upon CCL27 stimulation probably resulting in accelerated wound closure suggesting that keratinocytes may be beneficial for treatment of wounds where fast wound closure without increased stimulation of granulation tissue is needed (e.g. burn wounds). This is in line with clinical data in which keratinocytes are applied to close burn wounds without stimulating granulation tissue formation¹⁵.

In chapter 3 and 4 we further explored in detail the effect of CCL27 on ASC, dermal fibroblasts, keratinocytes and monocytes, human microvascular endothelial cells (hMVECs) and granulocytes. CCL27 is present in a wide variety of wound exudates and although its function with regards to wound healing is unknown, notably all of the studied cell types expressed the receptor for CCL27 (CCR10). In addition to expressing the CCR10 receptor, CCL27 is also secreted by keratinocytes, hMVECs and monocytes after stimulation with factors related to skin trauma. Next to secreting CCL27, keratinocytes did show increased proliferation and migration upon CCL27 exposure which indicates that an autocrine feedback loop may be involved in regulating this process. An autocrine feedback loop may be also involved in the regulation of the secretion of inflammatory mediators by monocytes. Although granulocytes, dermal fibroblasts and hMVEC did express CCR10 they did not respond significantly to CCL27. These results indicate that upon CCL27 exposure keratinocytes are primarily activated to promote wound closure and monocytes respond to secrete more inflammatory mediators (IL-6, CXCL1, CXCL8, CCL2 and CCL20) probably further amplifying the inflammatory response. In contrast ASC respond, next to secreting inflammatory mediators (CXCL1 and CXCL8), to increase secretion of angiogenic and granulation tissue formation factors (VEGF and IL-6), which in turn can result in increased production of extracellular matrix. Taken together these results highlight multi-functional roles for CCL27 in cutaneous wound healing.

Upon tissue injury a temporary fibrin matrix is formed that serves as a network for incoming cells (e.g. endothelial cells) and fibrin variants can influence the extent of neovascularization^{16,17}. Until neovascularization occurs, a hypoxic environment (< 5% oxygen) is present and this hypoxia may influence scar formation. In order to further elucidate the contribution of ASC in abnormal scar formation the influence of oxygen tension (hypoxia 1% and normoxia 20 % oxygen) and naturally occurring fibrinogen variants on ASC expansion and differentiation were determined in chapter 5. ASC cultured in 1% oxygen showed increased proliferation and reduced cell aging compared to ASC cultured in 20% oxygen. Differentiation of ASC towards adipogenic and osteogenic lineages was improved in 20% oxygen, whereas 1% oxygen improved chondrogenic differentiation. The various fibrinogen coatings did not influence ASC expansion and differentiation and therefore are likely not an improvement as a component of the dermal matrix for our hypertrophic scar model. In contrast, oxygen tension did alter ASC behaviour and more research is needed to determine how it may influence scar formation. Next our observation that ASC expansion and differentiation is not influenced by different fibrinogen coatings suggests that fibrinogen is a suitable matrix in combination with ASC for tissue engineering applications. Also hypoxic culture may be beneficial for the expansion and maintenance of ASC culture due to increased proliferation and reduced cell aging.

In this project two different hypertrophic scar models were developed. A major challenge is to develop an abnormal scar model that can distinguish between hypertrophic scars and keloids. In Chapter 6 an abnormal scar model derived from primary cells isolated directly from freshly excised scar tissue (normal skin, normotrophic scar, hypertrophic scar and keloid), is used to reconstruct 3D scars *in vitro*. In the tissue-engineered scar models we identified parameters which distinguish normal skin from normotrophic scars and abnormal scars. Both abnormal scar models showed increased dermal thickness, increased contraction and increased α -SMA staining compared to normal skin and normotrophic scar models. Vimentin staining was equally present in all models indicating that equal numbers of fibroblasts were present. These results taken together indicate that cells isolated from different scars maintain their intrinsic characteristics. The normotrophic scar model and hypertrophic scar model could further be distinguished by decreased secretion of inflammatory mediators (CCL5, CXCL1 and IL-6) in the hypertrophic scar model compared to the normotrophic scar model. Interestingly, we also found differences between *in vitro* hypertrophic scar and keloids models. One mediator was decreased (CCL5) in the hypertrophic scar model compared to keloid model and in contrast IL-18 was increased in hypertrophic scar model compared to keloid model. The gene ITGA5, involved in cell matrix adhesion, was increased in hypertrophic scar model compared to keloid model whereas MMP1 expression, involved in ECM degradation, was decreased in hypertrophic scar model compared to keloid model. These data clearly show that we could develop abnormal scar models of different types of scars distinguishing normal skin, normotrophic scar, hypertrophic scar and keloid. It indicates that cells isolated from scars maintain their intrinsic characteristics and that pathogenesis of keloid and hypertrophic scars is different. However, these *in vitro* models are dependent on excised scar tissue, which is limited.

Therefore the second hypertrophic scar model described in chapter 7 consisted of reconstructed epidermis on an ASC populated matrix. The cells used to construct this model were obtained from healthy adult abdominal skin obtained from routine surgical procedures which is therefore more readily available. Quantifiable parameters (contraction, collagen I secretion, α -SMA staining, epidermal thickness, outgrowth of epidermis, secretion of CXCL8 and IL6) were also identified and this model was selected for further investigation and validation with a test panel of therapeutics. Interestingly, each therapeutic selectively effected a different combination of parameters (e.g. 5-fluorouracil decreased contraction and epidermal thickness in contrast triamcinolone which decreased collagen I secretion and epidermal thickness), supporting clinical findings that combined therapies may be most beneficial¹⁸. Also, no therapeutic normalized all scar parameters. This correlates to human experience in which currently no single therapeutic is able to completely restore a hypertrophic scar phenotype to a normal skin

phenotype^{19,20}. ASC in combination with healthy keratinocytes can be used to generate an *in vitro* hypertrophic scar model. The model can now be used to select therapeutics which normalize different scar parameters and in this way to test the potential added benefit to the patient of a combination therapy.

Future directions

Despite ongoing research in the field of wound healing and scar formation there is no good therapy to prevent abnormal scar formation. The extrapolation of research results is complicated by non-optimal human *in vivo* like scar models. In chapter 8 we reviewed the progress, limitations and future challenges of tissue-engineered scar models. Although our scar models described in chapter 6 and 7 are a clear improvement, the models did not address important questions referring to why an abnormal scar forms instead of a normotrophic scar and what causes a hypertrophic scar to form rather than a keloid scar. Also the genetic pre-disposition of individuals and the involvement of the immune system is not yet taken into account and remain challenges for the future.

Researchers in the field of scar formation should keep in mind that cutaneous wound healing and scar formation is a dynamic process and depending on the study time point, different results can be found in different studies. For example in both normotrophic and hypertrophic scar formation macrophage infiltration was observed however infiltration occurred at a later time point in hypertrophic scar tissue compared to normotrophic scar tissue (chapter 2). In order to prevent incorrect interpretations, scar formation should be studied over a period of time rather than a single point in time.

Literature describes differences between normal and hypertrophic scar formation in almost all stages of the wound healing process (e.g. exaggerated inflammation, prolonged reepithelialisation, increased extracellular matrix production)^{4,19}. We showed that the inflammatory phase seems to be impaired by decreased expression of inflammatory mediators and delayed infiltration of macrophage type 2 cells in hypertrophic scar tissue compared to normotrophic scar tissue. Whether the decreased expression of inflammatory mediators also affects the infiltration of other immune cells (e.g. macrophage type 1, mast cells, T lymphocytes) should be studied in the future. The failure of immunosuppressive therapies to improve abnormal scar formation is partly explained by these findings. Instead of the current used anti-inflammatory scar therapies, immune stimulatory therapies may be a better strategy for improving scar quality and deserve attention in the future.

Adding an angiogenic component to the scar models may be a further improvement, because we showed that angiogenic genes are differently expressed during hypertrophic scar formation compared to normotrophic scar formation (chapter 3). This is further supported by an increased number of microvessels found in hypertrophic scars compared to normotrophic scars⁴. The fact that hypertrophic scars occur more often

after closure of deep 3rd degree burn wounds suggests that endothelial cells present in hypertrophic scars may have originated from adipose tissue. It may be interesting to determine whether adipose tissue derived endothelial cells play a role in hypertrophic scar formation.

Future research on hypertrophic and keloid scar formation may be accelerated by the multidisciplinary scientific field organ-on-a-chip. Organ-on-a-chip involves engineered tissues which closely mimic *in vivo* tissues. It consists of multiple different cell types adjacent to and interacting with each other under closely controlled conditions, and is importantly grown in a microfluidic chip. Such an approach will enable studying of the general pathophysiology of scar formation.

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